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Heteroallelic Missense Mutations of the Galactosamine-6-Sulfate Sulfatase (GALNS) Gene in a Mild Form of Morquio Disease (MPS IVA)

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Morquio disease (MPS IVA) is an autosomal recessive disorder caused by a deficiency of N-acetylgalactosamine-6-sulfate sulfatase (GALNS) activity. Patients commonly present in early infancy with growth failure, spondyloepiphyseal dysplasia, corneal opacification, and keratan sulfaturia, but milder forms have been described. We report on a patient who grew normally until age 5 years. Her keratan sulfaturia was not detected until adolescence, and she now has changes restricted largely to the axial skeleton. She has experienced only mildly impaired vision. At age 22, thin-layer chromatography of purified glycosaminoglycans showed some keratan sulfaturia. GALNS activity in fibroblast homogenate supernatants was 20 ± 5% of controls (as compared to 5 ± 3% of controls in severe MPS IVA, $P < .003$). Kinetic analysis of residual fibroblast GALNS activity in patient and parents revealed decreased K_m and increased V_{max} in the mother and daughter, but not in the father, compatible with compound heterozygosity. GALNS exons were amplified from patient genomic DNA and screened by SSCP. Two missense mutations, a C to T transition at position 335 (predicting R94C) and a T to G transversion at position 344 (predicting F97V), were found on sequencing an abnormally migrating exon 3 amplicon. Digestion of the amplicon with *Fok*I and *Acc*I restriction enzymes (specific for the R94C and F97V mutations, respectively) confirmed heterozygosity. In fibroblast transfection experiments, heterozygous R94C and F97V mutants independently expressed as severe and mild GALNS deficiency, respectively. We interpret these findings to indicate that our patient bears heteroallelic GALNS missense mutations, leading to GALNS deficiency and mild MPS IVA. Our findings expand the clinical and biochemical phenotype of MPS IVA, but full delineation of the genotype-phenotype relationship requires further study of native and transfected mutant cell lines.

KEY WORDS: spondyloepiphyseal dysplasia, mucopolysaccharidosis, keratan sulfate, galactosamine-6-sulfate sulfatase, Morquio syndrome

INTRODUCTION

Skeletal changes that predominantly affect epiphyseal growth plates and vertebrae may be associated with specific abnormalities in glycosaminoglycan degradation, particularly those of keratan sulfate. These disorders have been termed Morquio-Ullrich syndrome, but are more rationally grouped together as mucopolysaccharidosis type IV (MPS IV) [Chetley, 1993; Arbisser et al., 1977; Neufeld and Muenzer, 1995]. To date, defects in two of the enzymes responsible for keratan sulfate degradation have been linked to specific clinical phenotypes. In MPS IVA (MIM 253000), deficiency of N-acetylgalactosamine-6-sulfate sulfatase (GALNS) has been shown to be responsible for keratan sulfaturia and is associated with the distinctive clinical findings of early-onset dysostosis multiplex (predominantly spondyloepiphyseal dysplasia), corneal clouding, odontoid hypoplasia, and aortic valve disease. In

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MPS IVB (MIM 253010), β-galactosidase deficiency is also associated with keratan sulfaturia, but is characterized by a milder, late-onset form of spondyloepiphyseal dysplasia, corneal clouding, and findings consistent with glycolipid storage [Arbisser et al., 1977; Neufeld and Muenzer, 1995]. There are other mild clinical variants of Morquio-Ullrich syndrome [Beck et al., 1986; Nelson et al., 1988; Fang-Kircher et al., 1995], and phenocopies without keratan sulfaturia (MIM 252300) that remain unclassified [Byers et al., 1978; Toledo et al., 1978]. This report documents the clinical and biochemical features of a late-onset spondyloepiphyseal dysplasia and punctate corneal opacities in a patient with 20% residual enzymatic activity who was found to have heteroallelic missense mutations in exon 3 of her GALNS genes that expressed a partially functioning enzyme in cell culture.

CLINICAL REPORT

The patient was born by spontaneous vaginal delivery at term after an uneventful gestation and weighed 3,510 g. Her mother and father (age 22 and 24 years at time of conception) are not known to be consanguineous and are of predominantly British ancestry. They and 3 older sibs are entirely well, without skeletal deformities. The patient was hospitalized twice in the first year of life with lower respiratory tract infections; hypogammaglobulinemia was noted on both occasions. Chest radiographs taken at the time showed normal mineralization. No abnormalities of rib and vertebral architecture could be discerned. She was admitted to hospital at age 7 years with a 2-year history of progressively waddling gait. Because of inability to raise herself from a seated position, a primary neuromuscular disturbance was considered. However, results of further studies, including biopsies of liver and muscle, were normal.

Radiographs of the hips and pelvis taken at that time showed bilateral destructive changes in the acetabulae and femoral heads (Fig. 1a), but there was minimal evidence of platyspondyly (Fig. 1c) and her height was at the 25th centile (Fig. 2a). At age 9½ years, she was seen because of continued deterioration in gait and loss of mobility. Repeat radiographs showed progressive bilateral destruction of the femoral heads (Fig. 1b), flattening of the vertebrae with distinctive anterior wedging (Fig. 1d), and very mild changes in the limbs (Fig. 1e). The skull and ribs were not affected. On ophthalmoscopic examination, hyperopia and astigmatism were noted, but no corneal opacities were detected on slit-lamp examination. Toluidine blue staining of a periph-

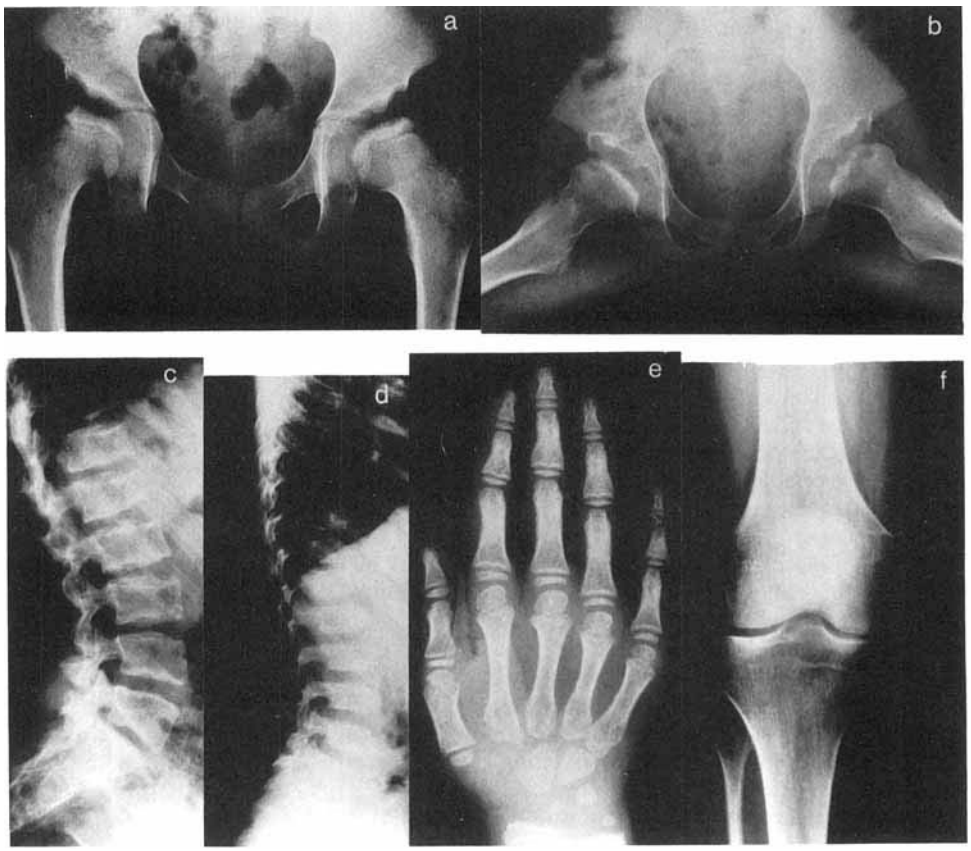

Fig. 1. Radiographic changes in mild Morquio disease. Comparison of the acetabula at age 7 (**a**) and age 9½ (**b**) underscore the rapid changes that occurred in the first few years after presentation. Although the vertebral bodies in the lower spine have a regular architecture at age 7 (**c**), they demonstrated distinctive anterior wedging at age 9½ (**d**). At the same time, however, the epiphyses of the hand at age 9½ were relatively unaffected (**e**), and the adult appendicular skeleton shows some osteopenia but normal architecture, as illustrated by the knee (**f**) at age 22.

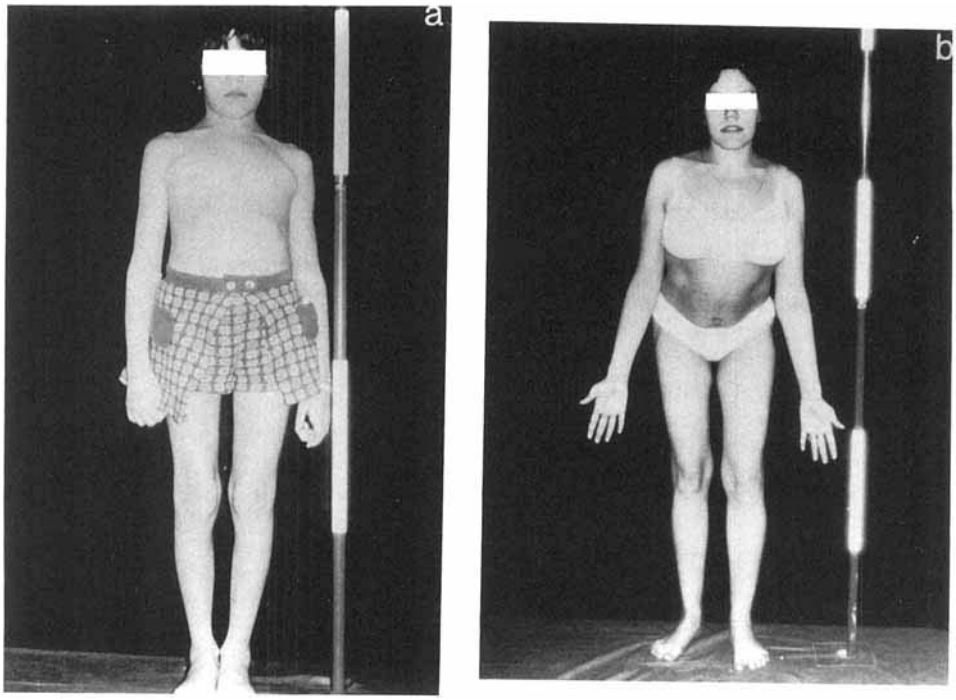

Fig. 2. Photographs of the patient at age 8 (**a**) and age 22 (**b**).

eral smear showed significant leukocytic metachromasia and, with Alcian blue staining, bone marrow histiocytes were said to be suggestive of a gangliosidosis. However, no keratan sulfate was detected on thin-layer chromatography of urine mucopolysaccharides, and the hexuronic acid excretion (4.4 mg/day) was not markedly raised. In an attempt to remedy her gait problems, a bilateral innominate osteoplasty was performed at age 9½ years, followed by a left hip fusion at age 13 years and right hip replacement at age 14 years. She continued to grow, but progressive kyphoscoliosis and axial deformities resulted in a mild continuous loss of height from a maximum of 151 cm at age 15 years.

She was seen for genetic counselling at age 18 regarding the risk of recurrence of her skeletal and eye problems in future offspring. Although her general health was good, she occasionally required walking aids because of the hip problems. She drove her own car and typed 50 words per min. By age 19, her height had declined to 149 cm with an arm span of 165 cm (upper/lower segment ratio of 0.76). Radiographic changes were still limited to her hips and spine, and the range of motion and skeletal architecture in her limbs (Fig. 1f) was otherwise normal. Ocular findings confirmed her high hyperopia, but also showed a diffuse opacification of both corneas throughout the entire stroma. The opacifications were round, discrete, and semiconfluent. Three years later, slit-lamp examination showed a slight increase in the density of these opacities, but there was no noticeable cloudiness of the cornea on routine clinical observation. At that time, electroretinography with oscillatory potentials was performed and found to be normal, as was a pattern voltage electroretinogram. The optic vitreous showed diffuse moderate syneresis. Diagnosis of a variant form of Morquio-Ullrich syndrome was considered, and she was counselled with regard to the low recurrence risk if her spouse had a negative family history.

When seen again at age 22 years (Fig. 2b), she had been married for 3 years to a nonconsanguineous 21-year-old man and was 5 months pregnant. At term, she delivered a healthy girl by cesarean section. Physical examination of her daughter, including careful slit-lamp examination at age 4 years, was unremarkable.

MATERIALS AND METHODS

MPS Screening

At 7, 9, and again at 18 years, multiple random and 24-hr urine collections at other centers had been subjected to toluidine blue and cetylpyridinium chloride precipitation screening tests without eliciting firm evidence of mucopolysaccharidosis. Investigation of a possible mucopolysaccharidosis was reinitiated at age 22.

Glycosaminoglycan Analysis

Glycosaminoglycans (GAG) were isolated from urine by precipitation with cetylpyridinium chloride [Gordon and Haust, 1970], and the mixed GAG was either chromatographed directly or digested with hyaluronidase, and then chromatographed on a cellulose thin layer plate [Humbel and Chamoles, 1972]. The hyaluronidase digestion involved incubation of 1 mg of the isolated urinary GAG mixture in 0.1 M acetate buffer, pH 4.75, overnight at 37°C (0.3 mg testicular hyaluronidase/ml incubate).

Lysosomal Enzyme Activities

Cultured dermal fibroblasts were grown from the patient, her family, and control subjects [Sergovich, 1976]. Activities of β-N-acetylhexosaminidases were assessed as previously reported [Gordon et al., 1988], while β-galactosidase and other acid hydrolases were assayed with fluorimetric substrates [Wenger and Williams, 1991] with minor modifications. GALNS sulfatase activities were measured using a radiolabeled trisaccharide substrate derived from chondroitin-6-sulfate [Glössel and Kresse, 1978]. The enzyme source in the assays of GalNAc-6-S sulfatase studies was a fibroblast homogenate supernatant. The fibroblast pellet was suspended in 300 μl of water, cycled through freeze-thaw cycles, and centrifuged at 16,000 rpm for 6 min and the supernatant was removed for use in the enzyme assay. The radioisotopic assay of GALNS activity was carried out in triplicate with 214 pmoles of N-acetylgalactosamine-6-sulfate-β-1, 4-glucuronyl-β-1, [3-^{3}H]-galactosaminol-6-sulfate, plus 15 μg of the crude enzyme protein, in 10 mM acetate buffer, pH 3.75, plus 0.15 mg/ml bovine serum albumin in a total volume of 75 μl. The digestion mixture was incubated at 37°C for 2 hr, and the desulfated product analyzed [Glössel and Kresse, 1978].

Northern Blot and Southern Blot Analysis

Total RNA was extracted by the guanidinium thiocyanate procedure [Chomczynski and Sacchi, 1987], and genomic DNA was obtained by a standard method [Sambrook et al., 1989]. Five micrograms of genomic DNA were digested by *BamHI* and electrophoresed in a 0.6% agarose gel. The DNA was transferred to Hybond-N membrane (Amersham, Buckinghamshire, UK) and hybridized with full-length GALNS cDNA [Tomatsu et al., 1991], labeled with [α-^{32}p]-dCTP in order to detect large rearrangements. Northern blotting was carried out by a glyoxal method [Sambrook et al., 1989]. Approximately 50 μg of total cellular RNA were blotted onto the Hybond-N. The filter was hybridized with full-length cDNA to look for a promoter mutation or transcriptional error of the GALNS gene.

Amplification of Genomic DNA by PCR

With knowledge of the sequence of 14 exon and intron junctions of the GALNS gene, we amplified all coding regions and flanking intron sequences by PCR [Saiki et al., 1985], incorporating the [α-^{32}P]-dCTP to label the product for SSCP analysis [Orita et al., 1989; Tomatsu et al., 1995a]. The primer sequences, PCR conditions, and SSCP screening strategy have been described elsewhere [Ogawa et al., 1995]. The specific fragment with abnormal SSCP mobility was amplified again, purified, and ligated into the T-vector (Novagen, Madison, WI) for fluorescent dideoxy cycle sequencing (Applied Biosystems, Foster City, CA). Five independent clones were sequenced.

Construction of Expression Vector and Transient Expression of Mutant Alleles

In order to construct the expression plasmid vector of the identified mutation, site-directed mutagenesis (CLONTECH, Palo Alto, CA) was used according to the supplier's recommendations. The primer sequence that eliminated the *Hind*III restriction site was 5′-TTCTATTACTGTGGCGAC-3′, and primer sequences of the unique R94C and F97V mutations (see Results) were 5′-GCTACCCATCTGCAATGGCTT-3′ and 5′-CCGCAATGGCGTCTACACCAC-3′, respectively. The sequence of the construct was confirmed by dideoxy sequencing. The R94C, F97V, and normal constructs of GALNS cDNA were transiently transfected by Gene Transfer™ (Wako, Osaka, Japan) into five dishes of enzyme-deficient fibroblasts established from a classical Morquio patient. The GALNS activity in the cell extract was measured 120 hr after transfection, using the labeled trisaccharide as substrate [Glössel and Kresse, 1978; Masue et al., 1991; Tomatsu et al., 1991].

RESULTS

GAG Studies

GAGs from the patient's urine on thin-layer chromatography (Fig. 3) contained a keratan sulfate band running close to the front which was similar to that present in a known MPS IV urine (positive control). The chondroitin sulfate present in the positive and negative controls and our patient's urine was susceptible to hyaluronidase digestion, but the keratan sulfate band was unchanged. The keratan sulfate was present in both the pregnant and nonpregnant urine collected from our patient.

Enzyme Studies

In cultured fibroblast homogenates from our patient and her parents, there was normal activity of β-galactosidase, α-mannosidase, α-fucosidase, hexosaminidase, and β-glucuronidase enzymes. In the supernatants of fibroblast homogenates from the patient, GALNS activity was significantly reduced to <20% of the normal control mean (Table I). Under the same con-

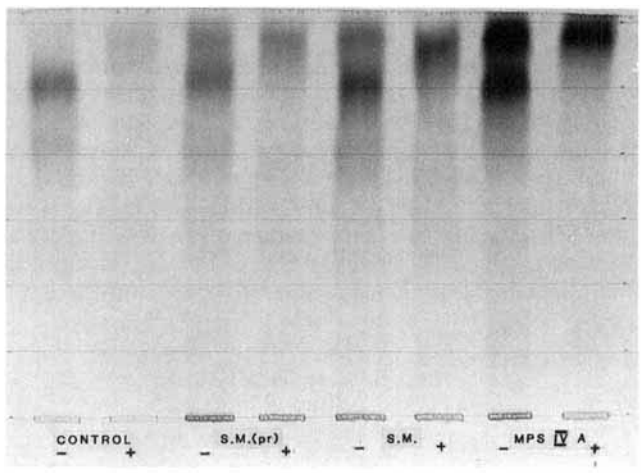

Fig. 3. Thin-layer chromatograms of urine GAG. The urine GAG mixtures were spotted before (−) or after (+) hyaluronidase digestion. The urinary GAGs, spotted from left to right, include those from a control, from our patient while pregnant (pr), from our patient following her pregnancy, and from a known MPS IVA patient.

TABLE I. GALNS Activity in Native Fibroblasts (pmol/mg protein/hr)

Subject	Genotype	Native activity (n)	Percentage of control
Normal controls		1,122 ± 210 (11)[a]	100
Proposita	R94C/F97V	220 ± 56 (5)[b,] *	20
Mother	R94C/+	268 ± 55 (4)*	24
Father	F97V/+	843 ± 240 (5)*	74
Daughter	F97V/+	706 ± 8 (4)*	62
Husband	+/+	1,030 ± 124 (4)	90
Classical MPS IV		62 ± 36 (5)*	5

[a]Samples from 11 control fibroblast lines.
[b]Replicates for proband and family members.
*Significant difference ($P < 0.05$) from normal controls, Mann-Whitney test.

ditions, fibroblast homogenate supernatants from a classical MPS IVA patient showed 5% or less of the control mean. Enzyme activity in the mother, an obligate heterozygote, was also significantly reduced ($P < 0.005$) to 24% of control, while levels in the patient's father and daughter, also heterozygotes, were intermediate (74% and 62%, respectively).

Because there was significant residual activity in the proband fibroblasts, saturation kinetics could be examined. Shown in Table II are the affinity constant (K_M) and the maximal velocity (V_{max}) along with results from 3 controls. Again, there is a marked reduction in V_{max} for the heterozygous maternal fibroblasts similar to values in the proposita, while values for the father are not distinguishable from controls. The K_M or half-saturation constant is also decreased in both the mother and proposita more than in the father.

Southern and Northern Blot Analysis

No abnormal band was present on genomic Southern blot, suggesting there was no gross alteration of the proposita's GALNS alleles. The mRNA of the patient was equivalent in size and abundance to the control sample, indicating there was no mutation affecting the RNA transcript.

SSCP and Sequence of PCR Fragment

An abnormal band was detected in the PCR product of exon 3 by SSCP analysis. PCR products from other exons did not show abnormal mobilities. Sequencing of the novel exon 3 fragment revealed two mutant alleles. One was a C to T transition at nucleotide 335 that replaced Arg 94 with Cys (designated R94C). The other was a T to G transversion at nucleotide 344 that led to Phe at position 97 being replaced by Val (designated F97V). Out of five clones sequenced, two contained only R94C and three only F97V. Neither the normal sequence nor clones harboring both mutations on one strand were found in the PCR product of the patient's exon 3. In order to exclude an artifact of the Taq DNA polymerase and confirm authenticity of these mutations, PCR amplicons of exon 3 from the patient and her family were subjected to restriction enzyme analysis. When the R94C mutation occurred, a *Fok*I site was destroyed by replacing the restriction site 5′-NNNNNNNNNNNNCATCC-3′ with 5′-NNNNNNNNNNNNNCATCT-3′. As shown in Figure 4a, only the patient and her mother had digested and undigested bands after *Fok*I treatment, demonstrating maternal inheritance of the R94C mutation. On the other hand, the F97V mutation generated a new *Acc*I restriction site (TTCTAC→GTCTAC). Figure 4b shows the *Acc*I analysis for the F97V mutation. The patient, her father, and her daughter had the digested and undigested bands, indicating paternal transmission of the F97V mutation.

TABLE II. Kinetics of GALNS in Native Cultured Fibroblasts

	K_M (μmol/l)	V_{max} (pmol × mg protein^{-1} × hr^{-1})
Controls (n = 3)	60 ± 20	4.7 ± 1.8
Mother	29	2.0
Father	50	4.0
Patient	33	1.0

Transient Expression of Mutant R94C and F97V Alleles in Enzyme-Deficient Fibroblasts

Cells transfected with either the R94C or the F97V construct showed less GALNS activity, as compared with cells transfected with normal GALNS cDNA (Table III). Activity of the R94C GALNS was only 1% of normal, which is about the same as seen in classical MPS IVA-transfectant cell lines. The F97V was about 14% of the untransfected cell line, while cells cotransfected with both mutant alleles had only slightly less (12% activity).

DISCUSSION

Our patient, a short 22-year-old woman with late-onset but progressive spondyloepiphyseal dysplasia and corneal opacifications, presents with a clinical picture consistent with mild or juvenile-onset Morquio-Ullrich syndrome. Although evidence of defective keratan sulfate catabolism was demonstrated and β-galactosidase activity was normal, she retained appreciable levels of residual activity GALNS when her fibroblasts were assayed with a radioactive chondroitin-6-sulfate-derived trisaccharide substrate.

Similar mild variants of the MPS IV syndrome with GALNS deficiency have been reported previously by Glössl et al. [1981], case 1; Orii et al. [1981], 2 sibs;

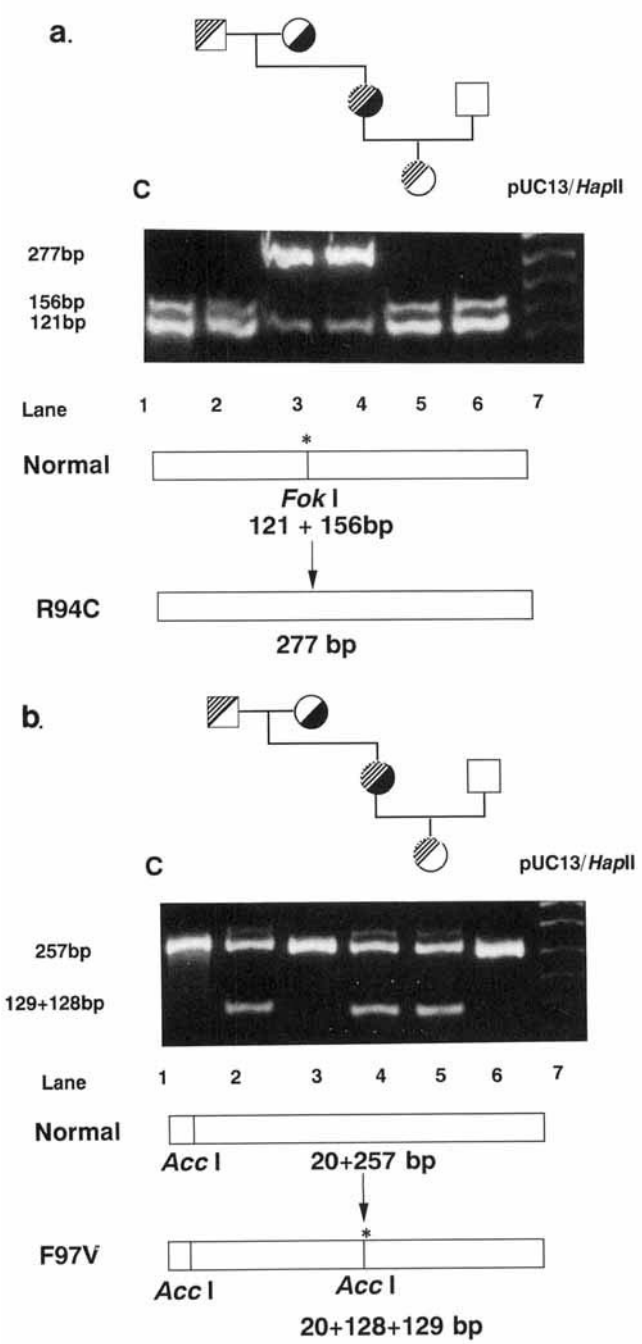

Fig. 4. Restriction enzyme analysis performed on the PCR fragment of exon 3. **a:** *FokI* analysis of the R94C mutation. The digested 156 + 122-base pair (bp) bands and the undigested 277-bp band indicate normal and R94C allele, respectively. All individuals had the digested normal allele, but the proposita and her mother carried the R94C allele, demonstrating that they are heterozygous for the R94C mutation. **b:** *AccI* analysis of the F97V mutation. The larger 257-bp band represents a normal allele, while the smaller 129 + 128-bp fragments are products of the F97V allele. The father, the proposita and her daughter had both the 128 + 127-bp and 257-bp bands, demonstrating they are heterozygous with F97V. The 129-bp and 128-bp bands are indistinguishable, and the smallest 20-bp band is not seen in this gel.

Fujimoto and Horowitz [1983], 2 sibs; Hecht et al. [1984], 1 case; Beck et al. [1986]; Nelson et al. [1988], cases 3, 4, 5, 9, and 10; and Fang-Kircher et al. [1995], 1 case. However, the residual enzyme activities assayed in the patient fibroblasts have not generally distinguished the "mild" patients from those with the classical form of disease and severe clinical manifestations. When Nelson et al. [1988] compared residual enzyme activities in fibroblasts from 12 Morquio patients with either mild or classical forms of the disease, using the chondroitin-6-sulfate-derived trisaccharide as substrate, activity in all but one of the patients was said to be <10% of the control. One of those with mild clinical disease apparently had 30% of the activity encountered in control cells. The pH profile, K_M, and thermal stability of the residual enzyme in this patient's cells were not different from normal [Nelson et al., 1988]. Glössl et al. [1981] and Beck et al. [1986] reported mutant enzymes with a shift in the pH optimum to a more acidic pH than controls. The K_M of the mutant enzyme in the patient fibroblasts described by Glössl et al. [1981] was similar to that of the control enzyme; however, the mutant enzyme, like the enzyme from fibroblasts of a severe Morquio patient, was abnormally thermolabile. In the adult studied by Fang-Kircher et al. [1995], no abnormalities were detected until adulthood although the enzyme activity was less than 16% of normal and thin-layer chromatography did not demonstrate any abnormality.

In our patient's family, the differences in residual GALNS enzyme activity and kinetics, as manifested by the parental cell lines, suggest that they were contributing different mutant alleles. Molecular analysis of the patient's GALNS cDNA confirmed the presence of two mutant alleles, R94C and F97V, both in exon 3. Although a third cryptic mutation cannot be ruled out, the entire GALNS coding sequence of the patient, except for exon 3, was normal; R94C and F97V were the only changes detected. The maternal R94C mutation appears to cause a severe loss of enzyme activity in cultured fibroblasts and is associated with significant changes in enzyme kinetics, also seen in the activity of R94C-transfected fibroblasts. In contrast, there is a high residual activity in cells from the proposita's father and daughter (72% and 62%, respectively), matched by significant residual activity (14%) in F97V-transfected enzyme-depleted fibroblasts. This is unusual, in that other mutant GALNS alleles, expressed clinically as mild Morquio syndrome (N204K and V138A), show virtually no detectable enzyme activity in vitro [Fukuda et al., 1992; Ogawa et al., 1995]. The higher activity of the F97V transfectant may be explained by the fact that the Phe97 residue is not located in any of the domains evolutionarily conserved in the sulfatase family of genes [Tomatsu et al., 1991]. Moreover, the substitution of phenylalanine by valine is a relatively conservative change that would preserve hydrophobicity in the domain of the expressed protein. In keeping with this interpretation is the decreased enzyme velocity and decreases in the half-saturation constants found in the mutant fibroblast kinetic experiments. These are the sorts of changes one might expect if the mutations did not interfere with the binding of substrate. If native GALNS is a homodimer (molecular mass ~120 kDa [Masue et al., 1991]), it follows that the

TABLE III. GALNS Activity in Transfected Fibroblasts (pmol/mg protein/hr)

Transfection	Genotype	GALNS activity (n)[a]	Percentage[b]
Untransfected		16,300 ± 2,200 (3)	100
MPS IVA[c]	del/del	380 ± 250 (3)	2
R94C	+/R94C	190 ± 430 (5)	1
F97V	+/F97V	2,320 ± 970 (5)	14
R94C/F97V cotransfection	R94C/F97V	1,990 ± 340 (3)	12
Normal	+/+	21,100 ± 4,800 (5)	129

[a]Number of replicates for transfection and enzyme assay.
[b]Activity relative to the untransfected cell line.
[c]Cells used in the transfection experiment were established from a classical Morquio patient harboring large structural changes in both GALNS alleles. Absence of mRNA transcript and mutant protein were established by Northern and Western blot analysis, respectively.

kinetic data could be explained by abnormal subunit interactions. Heterozygotes would be expected to have varying fractions of normal, mixed, and mutant dimers, and the proband would be expected to express varying fractions of three mutant dimers, i.e., F97V/F97V protein, R94C/R94C protein, and F97V/R94C protein. In the transfection experiments, the F97V mutant and the R94C/F97V mutant demonstrated similar enzymatic activity, as measured with the N-acetylated heterotrisaccharide, suggesting that F97V activity is expressed dominantly, relative to R94C, but codominantly with the normal allele. However, R94C activity is dominant to the normal allele in vitro, leaving unexplained the normal maternal phenotype. Another important consideration is the potential differences in mutant and normal enzyme activity toward a second natural GALNS substrate, the galactose-6-sulfate residues of keratan sulfate. The maternal R94C enzyme complex may be more active toward keratan sulfate, and such differences would not be detected in our biochemical assay. It is clear that further experiments are needed to delineate the relative roles of N-acetylgalactosamine-6-sulfate and galactose-6-sulfate sulfatase activities in normal and abnormal glycosaminoglycan metabolism of cartilage and bone.

Among the more than 50 MPS IVA patients studied, 40 mutations have so far been identified [Tomatsu et al., 1995b], and no other F97V mutations have been found. On the other hand, R94C has been detected in one other unrelated heteroallelic patient [Ogawa et al., 1995]. Unfortunately, clinical information is not available for that patient, so that the effect of this mutation on phenotype remains to be analyzed.

In conclusion, this case report reinforces the clinical point that keratan sulfaturia is not a constant finding in MPS IV patients of various ages [Hecht et al., 1984]. The ability to demonstrate keratan sulfate as an abnormal urinary constituent is critically dependent on the method employed. In a review of appropriate analytical procedures, Longdon and Pennock [1979] suggested that either thin-layer chromatography of the isolated GAG, as used in the present study, or two-dimensional electrophoresis [Nelson et al., 1988] must be used to demonstrate keratan sulfaturia. Our case expands the spectrum of mild MPS IVA and suggests that care must be taken in excluding this diagnosis on the basis of negative screening tests [Dembure, 1992].

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